

APPLICATION  
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TITLE: MODIFIED VITAMIN K-DEPENDENT POLYPEPTIDES  
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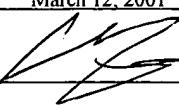
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## MODIFIED VITAMIN K-DEPENDENT POLYPEPTIDES

### Cross Reference to Related Applications

INS A1 > This application is a continuation-in-part of U.S. Serial No. 08/955,636, filed on  
5 October 23, 1997.

### Statement as to Federally Sponsored Research

Funding for work described herein was provided by the federal government, which has certain rights in the invention.

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### Background of the Invention

Vitamin K-dependent proteins contain 9 to 13 gamma-carboxyglutamic acid residues (Gla) in their amino terminal 45 residues. The Gla residues are produced by enzymes in the liver that utilize vitamin K to carboxylate the side chains of glutamic acid residues in protein precursors. Vitamin K-dependent proteins are involved in a number of biological processes, of which the most well-described is blood coagulation (reviewed in Furie, B. and Furie, B.C., 1988, Cell, 53:505-518). Vitamin K-dependent proteins include protein Z, protein S, prothrombin, factor X, factor IX, protein C, factor VII and Gas6. The latter protein functions in cell growth regulation. Matsubara et al., 1996, Dev. Biol., 180:499-510. The Gla residues are needed for proper calcium binding and membrane interaction by these proteins. The membrane contact site of factor X is thought to reside within amino acid residues 1-37. Evans and Nelsestuen, 1996, Protein Science 5:suppl. 1, 163 Abs. Although the Gla-containing regions of the plasma proteins show a high degree of sequence homology, they have at least a 1000-fold range in membrane affinity. McDonald, 25 J.F. et al., 1997, Biochemistry, 36:5120-5137.

Factor VII functions in the initial stage of blood clotting and may be a key element in forming blood clots. The inactive precursor, or zymogen, has low enzyme activity that is greatly increased by proteolytic cleavage to form factor VIIa. This activation can be catalyzed by factor Xa as well as by VIIa-tissue factor, an integral membrane protein found 30 in a number of cell types. Fiore, M.M., et al., 1994, J. Biol. Chem., 269:143-149. Activation

by VIIa-tissue factor is referred to as autoactivation. It is implicated in both the activation (formation of factor VIIa from factor VII) and the subsequent activity of factor VIIa. The most important pathway for activation *in vivo* is not known. Factor VIIa can activate blood clotting factors IX and X.

5           Tissue factor is expressed at high levels on the surface of some tumor cells. A role for tissue factor, and for factor VIIa, in tumor development and invasion of tissues is possible. Vrana, J.A. et al., Cancer Res., 56:5063-5070. Cell expression and action of tissue factor is also a major factor in toxic response to endotoxic shock. Dackiw, A.A. et al., 1996, Arch. Surg., 131:1273-1278.

10           Protein C is activated by thrombin in the presence of thrombomodulin, an integral membrane protein of endothelial cells. Esmon, N.L. et al., 1982, J. Biol. Chem., 257:859-864. Activated protein C (APC) degrades factors Va and VIIa in combination with its cofactor, protein S. Resistance to APC is the most common form of inherited thrombosis disease. Dahlback, B., 1995, Blood, 85:607-614. Vitamin k inhibitors are commonly 15 administered as a prophylaxis for thrombosis disease.

              Vitamin k-dependent proteins are used to treat certain types of hemophilia. Hemophilia A is characterized by the absence of active factor VIII, factor VIIIa, or the presence of inhibitors to factor VIII. Hemophilia B is characterized by the absence of active factor IX, factor IXa. Factor VII deficiency, although rare, responds well to factor VII 20 administration. Bauer, K.A., 1996, Haemostasis, 26:155-158, suppl. 1. Factor VIII replacement therapy is limited due to development of high-titer inhibitory factor VIII antibodies in some patients. Alternatively, factor VIIa can be used in the treatment of hemophilia A and B. Factor IXa and factor VIIIa activate factor X. Factor VIIa eliminates the need for factors IX and VIII by activating factor X directly, and can overcome the 25 problems of factor IX and VIII deficiencies with few immunological consequences. Hedner et al., 1993, Transfus. Medi. Rev., 7:78-83; Nicolaisen, E.M. et al., 1996, Thromb. Haemost., 76:200-204. Effective levels of factor VIIa administration are often high (45 to 90  $\mu$ g/kg of body weight) and administration may need to be repeated every few hours. Shulman, S. et al., 1996, Thromb. Haemost., 75:432-436.

A soluble form of tissue factor (soluble tissue factor or sTF) that does not contain the membrane contact region, has been found to be efficacious in treatment of hemophilia when co-administered with factor VIIa. U.S. Patent No. 5,504,064. In dogs, sTF was shown to reduce the amount of factor VIIa needed to treat hemophilia. Membrane association by 5 sTF-VIIa is entirely dependent on the membrane contact site of factor VII. This contrasts to normal tissue-factor VIIa complex, which is bound to the membrane through both tissue factor and VII(a).

### Summary of the Invention

10 It has been discovered that modifications within the  $\gamma$ -carboxyglutamic acid (GLA) domain of vitamin K-dependent polypeptides enhance their membrane binding affinities. Vitamin K-dependent polypeptides modified in such a manner have enhanced activity and may be used as anti-coagulants, pro-coagulants or for other functions that utilize vitamin k-dependent proteins. For example, an improved factor VII molecule may provide 15 several benefits by lowering the dosage of VIIa needed, the relative frequency of administration and/or by providing qualitative changes that allow more effective treatment of deficiency states.

The invention features vitamin k-dependent polypeptides that include a modified GLA domain that enhances membrane binding affinity of the polypeptide relative to a 20 corresponding native vitamin k-dependent polypeptide. The modified GLA domain is from about amino acid 1 to about amino acid 45 and includes at least one amino acid substitution. For example, the amino acid substitution can be at amino acid 11, 12, 29, 33 or 34. Preferably, the substitution is at amino acid 11, 33, or 34. The modified GLA domain may include an amino acid sequence which, in the calcium saturated state, forms a tertiary 25 structure having a cationic core with a halo of electronegative charge.

The vitamin k-dependent polypeptide may be, for example, protein C, activated protein C, factor IX, factor IXa, factor VII, factor VIIa or active site modified factor VIIa. The modified GLA domain of protein C or activated protein C may include a glutamic acid residue at amino acid 33 and an aspartic acid residue at amino acid 34. The modified GLA

domain of protein C or activated protein C may also include a glutamine or glutamic acid residue at amino acid 11. Additionally, a glycine residue may be substituted at amino acid 12 in the GLA domain of protein C or activated protein C.

The modified GLA domain of factor VII, factor VIIa, active site modified factor VIIa, factor IX, and factor IXa may contain a substitution at amino acid 11, 29, 33, or combinations thereof. For example, the modified GLA domain may contain substitutions at residues 11 and 29, 11 and 33, 29 and 33, or 11, 29, and 33. The modified GLA domain can contain, for example, a substitution of a glutamine, a glutamic acid, an aspartic acid, or an asparagine residue at residue 11, and further can include a substitution at residue 29 such as substitution of a glutamic acid or a phenylalanine residue or an amino acid substitution at residue 33 such as a glutamic acid or an aspartic acid residue. The modified GLA domain can include a substitution of an aspartic acid residue at residue 33. Substitution of a glutamine residue at residue 11 is particularly useful. For example, a glutamine residue at residue 11 and a glutamic acid residue at residue 33 or a phenylalanine at residue 29 may be substituted. The GLA domain can include, for example, a substitution of a glutamic acid or a phenylalanine residue at residue 29 and further can include a substitution of a glutamic acid or an aspartic acid at residue 33. Such a polypeptide further can include a glutamic acid or an aspartic acid residue at amino acid 33.

Isolated nucleic acid molecules that include a nucleic acid sequence encoding modified vitamin K-dependent polypeptides also are described. The nucleic acid molecules encode vitamin K-dependent polypeptides that include a modified GLA domain that enhances membrane binding affinity of the polypeptide relative to a corresponding native vitamin K-dependent polypeptide. The modified GLA domain of such encoded polypeptides can include a substitution at amino acid 11, 29, or 33 as discussed above. The native vitamin K-dependent polypeptide can be, for example, factor VII, factor VIIa, active-site modified factor VIIa, factor IX, or factor IXa.

The invention also features a mammalian host cell that includes a vitamin k-dependent polypeptide. The polypeptide includes a modified GLA domain that enhances membrane binding affinity of the polypeptide relative to a corresponding native vitamin k-

dependent polypeptide. The modified GLA domain includes at least one amino acid substitution at, for example, amino acid 11, 12, 29, 33 or 34. The vitamin k-dependent polypeptide may be, for example, factor VII or factor VIIa.

The invention also relates to a pharmaceutical composition that includes a pharmaceutically acceptable carrier and an amount of a vitamin k-dependent polypeptide effective to inhibit clot formation in a mammal. The vitamin k-dependent polypeptide includes a modified GLA domain that enhances membrane binding affinity of the polypeptide relative to a corresponding native vitamin k-dependent polypeptide. The modified GLA domain includes at least one amino acid substitution. The vitamin k-dependent polypeptide may be, for example, protein C, activated protein C or active site modified factor VIIa.

The invention also features a pharmaceutical composition that includes a pharmaceutically acceptable carrier and an amount of a vitamin k-dependent polypeptide effective to increase clot formation in a mammal. The vitamin k-dependent polypeptide includes a modified GLA domain that enhances membrane binding affinity of the polypeptide relative to a corresponding native vitamin k-dependent polypeptide. The modified GLA domain includes at least one amino acid substitution. For example, residue 11, 29, or 33 can be modified as discussed above. The vitamin k-dependent polypeptide may be, for example, factor VII, factor VIIa, factor IX or factor IXa. The pharmaceutical composition also may include soluble tissue factor. Such pharmaceutical compositions can be used to treat a bleeding disorder in a patient by administering the pharmaceutical composition to the patient.

A method of decreasing clot formation in a mammal is also described. The method includes administering an amount of a vitamin k-dependent polypeptide effective to decrease clot formation in the mammal. The vitamin k-dependent polypeptide includes a modified GLA domain that enhances membrane binding affinity of the polypeptide relative to a corresponding native vitamin k-dependent polypeptide. The modified GLA domain includes at least one amino acid substitution. The vitamin k-dependent polypeptide may be, for example, protein C, activated protein C or active site modified factor VIIa.

The invention also features a method of increasing clot formation in a mammal. The method includes administering an amount of a vitamin k-dependent polypeptide effective to increase clot formation in the mammal. The vitamin k-dependent polypeptide includes a modified GLA domain that enhances membrane binding affinity of the 5 polypeptide relative to a corresponding native vitamin k-dependent polypeptide. The modified GLA domain includes at least one amino acid substitution. For example, the modified GLA domain can include an amino acid substitution at residue 11, 29, or 33, as discussed above. The vitamin k-dependent polypeptide may be, for example, factor VII, factor VIIa, factor IX or factor IXa.

10 Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used to practice the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein 15 are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

20

#### Brief Description of the Drawings

Figure 1 depicts the binding, with standard deviations, of wild type VIIa (open circles), VIIQ11E33 (filled circles), and bovine factor X (filled triangles) to membranes.

Figure 2 depicts the autoactivation of VIIQ11E33. The dashed line shows activity 25 in the absence of phospholipid.

Figure 3 depicts the activation of factor X by factor VIIa. Results for wild type factor VIIa (open circles) and VIIaQ11E33 (filled circles) are given for a concentration of 0.06 nM.

Figure 4 depicts the coagulation of human plasma by VIIa and VIIaQ11E33 with soluble tissue factor.

Figure 5 depicts the coagulation of plasma by factor VII zymogens and normal tissue factor.

5 Figure 6 depicts the inhibition of clot formation by active-site modified factor VIIaQ11E33 (DEGR-VIIaQ11E33).

Figure 7 depicts the circulatory time of factor VIIQ11E33 in rats.

Figure 8 depicts the membrane interaction by normal and modified proteins.

Panel A shows the interaction of wild type bovine protein C (open circles) and bovine protein  
10 C-H11 (filled circles) with vesicles. Panel B shows the interaction of wild type human  
protein C (open circles) and human protein C-P11 (filled circles) with membranes. In both  
cases, the dashed line indicates the result if all of the added protein were bound to the  
membrane.

15 Figure 9 depicts the influence of activated protein C on clotting times. In panel A,  
the average and standard deviation for three determinations of clotting times for bovine  
plasma are shown for wild type bovine APC (open circles) and for bAPC-H11 (filled circles).  
In panel B, the average and standard deviation of three replicates of human plasma  
coagulation for the wild type human (open circles) and human APC-P11 (filled circles) are  
shown.

20 Figure 10 depicts the inactivation of factor Va by bovine and human APC. Panel  
A depicts the inactivation of factor Va by wild type bovine APC (open circles) and bovine  
APC-H11 (filled circles). Panel B depicts the inactivation of human factor Va in protein S-  
deficient plasma by either wild type human APC (open circles) and human APC-H11 (filled  
circles).

25 Figure 11 depicts the electrostatic distribution of protein Z. Vertical lines denote  
electropositive regions and horizontal lines denote electronegative regions

Figure 12 depicts the membrane binding and activity of various protein Cs. Panel  
A shows membrane binding by wild type protein C (open circles), the P11H mutant of  
protein C (filled squares), Q33E,N34D mutant (filled circles) and bovine prothrombin (open

squares). Panel B shows inhibition of blood coagulation by these mutants. Panel C shows the inactivation of factor Va.

Figure 13 compares membrane binding and activity of human protein C mutants. Panel A compares the membrane binding of wild-type (open circles), E33 (open triangles) and E33D34 (filled circles). Panel B compares the coagulation times using wild-type (open triangles), E33 (open circles) and E33D34 (filled circles).

Figure 14 compares membrane binding (Panel A) and coagulation inhibition (Panel B) with wild-type (open squares), H11 (filled circles), E33D34 (open triangles) and the triple H11E33D34 mutant (open circles) of bovine protein C.

Figure 15 depicts the membrane interaction properties of different vitamin K-dependent proteins. Panel A compares membrane interaction of human (filled circles) and bovine (open circles) factor X. Panel B shows membrane interaction by normal bovine prothrombin fragment 1 (open circles), fragment 1 modified with TNBS in the absence of calcium (filled circles) and fragment 1 modified with TNBS in the presence of 25 mM calcium (filled squares). Panel C shows the rate of protein Z binding to vesicles at pH 9 (filled circles) and 7.5 (open circles).

#### Detailed Description

In one aspect, the invention features a vitamin k-dependent polypeptide including a modified GLA domain with enhanced membrane binding affinity relative to a corresponding native vitamin k-dependent polypeptide. Vitamin k-dependent polypeptides are a group of proteins that utilize vitamin k in their biosynthetic pathway to carboxylate the side chains of glutamic acid residues in protein precursors. The GLA domain contains 9-13  $\gamma$ -carboxyglutamic acid residues in the N-terminal region of the polypeptide, typically from amino acid 1 to about amino acid 45. Protein Z, protein S, factor X, factor II (prothrombin), factor IX, protein C, factor VII and Gas6 are examples of vitamin k-dependent polypeptides. Amino acid positions of the polypeptides discussed herein are numbered according to factor IX. Protein S, protein C, factor X, factor VII and human prothrombin all have one less amino acid (position 4) and must be adjusted accordingly. For example, actual position 10 of

bovine protein C is a proline, but is numbered herein as amino acid 11 for ease of comparison throughout. As used herein, the term "polypeptide" is any chain of amino acids, regardless of length or post-translational modification. Amino acids have been designated herein by standard three letter and one letter abbreviations.

5 Modifications of the GLA domain include at least one amino acid substitution. The substitutions may be conservative or non-conservative. Conservative amino acid substitutions replace an amino acid with an amino acid of the same class, whereas non-conservative amino acid substitutions replace an amino acid with an amino acid of a different class. Non-conservative substitutions may result in a substantial change in the  
10 hydrophobicity of the polypeptide or in the bulk of a residue side chain. In addition, non-conservative substitutions may make a substantial change in the charge of the polypeptide, such as reducing electropositive charges or introducing electronegative charges. Examples of non-conservative substitutions include a basic amino acid for a non-polar amino acid, or a polar amino acid for an acidic amino acid. The amino acid substitution may be at amino acid  
15 11, 12, 29, 33, or 34. Preferably, the amino acid substitution is at amino acid 11, 33, or 34. The modified GLA domain may include an amino acid sequence which, in the calcium saturated state, contributes to formation of a tertiary structure having a cationic core with a halo of electronegative charge. Without being bound by a particular theory, enhanced membrane affinity may result from a particular electrostatic pattern consisting of an  
20 electropositive core completely surrounded by an electronegative surface.

Many vitamin K-dependent polypeptides are substrates for membrane-bound enzymes. Since no vitamin K-dependent polypeptides display the maximum potential membrane-binding affinity of a GLA domain, all must contain amino acids whose purpose is to reduce binding affinity. Consequently, many vitamin K-dependent polypeptides contain  
25 amino acids that are non-optimal from the standpoint of maximum affinity. These residues effectively disrupt the binding site to provide a more rapid turnover for an enzymatic reaction.

Lowered membrane affinity may serve several purposes. High affinity is accompanied by slow exchange, which may limit reaction rates. For example, when the

prothrombinase enzyme is assembled on membranes with high affinity for substrate, protein exchange from the membrane, rather than enzyme catalysis, is the limiting. Lu, Y. and Nelsetuen, G.L., 1996, Biochemistry, 35:8201-8209. Alternatively, adjustment of membrane affinity by substitution with non-optimum amino acids may balance the 5 competing processes of procoagulation (factor X, IX, VII and prothrombin) and anticoagulation (protein C, S). Although membrane affinities of native proteins may be optimal for normal states, enhancement of membrane affinity can produce proteins that are useful for *in vitro* study as well as improved therapeutics for regulating blood clotting in pathological conditions *in vivo*.

10 Various examples of GLA domain modified vitamin k-dependent polypeptides are described below.

The vitamin k-dependent polypeptide may be protein C or activated protein C (APC). Amino acid sequences of the wild-type human (hC) and bovine (bC) protein C GLA domain are shown in Table 1. X is a Gla or Glu residue. In general, a protein with neutral 15 (e.g., Q) or anionic residues (e.g., D,E) at positions 11, 33 and 34 will have higher membrane affinity.

**TABLE 1**

20 hC: ANS-FLXXLRH<sub>11</sub>SSLXRXCIXX<sub>21</sub>ICDFXXAKXI<sub>31</sub>FQNVDDTLAF<sub>41</sub>WSKH (SEQ ID NO:1)

bC: ANS-FLXXLRP<sub>11</sub>GNVXRXCSXX<sub>21</sub>VCXFXXARXI<sub>31</sub>FQNTXDTMAF<sub>41</sub>WSFY (SEQ ID NO:2)

The modified GLA domain of protein C or APC may include, for example, a 25 glutamic acid residue at amino acid 33 and an aspartic acid residue at amino acid 34. The glutamic acid at position 33 may be further modified to  $\gamma$ -carboxyglutamic acid *in vivo*. For optimum activity, the modified GLA domain may include an additional substitution at amino acid 11. For example, a glutamine residue may be substituted at amino acid 11 or alternatively, a glutamic acid or an aspartic acid residue may be substituted. A histidine

residue may be substituted at amino acid 11 in bovine protein C. A further modification can include a substitution at amino acid 12 of a glycine residue for serine. Replacement of amino acid 29 by phenylalanine, the amino acid found in prothrombin, is another useful modification. Modified protein C with enhanced membrane binding affinity may be used in 5 place of other injectable anticoagulants such as heparin. Heparin is typically used in most types of surgery, but suffers from a low efficacy/toxicity ratio. In addition, modified protein C with enhanced membrane affinity may be used in place of oral anticoagulants in the coumarin family, such as warfarin.

These modifications can also be made with active site modified APC. The active 10 site of APC may be inactivated chemically, for example, by N-dansyl-glutamyl glycylarginylchloromethylketone (DEGR) or by site-directed mutagenesis of the active site. Sorensen, B.B. et al., 1997, *J. Biol. Chem.*, 272:11863-11868. Active site-modified APC functions as an inhibitor of the prothrombinase complex. Enhanced membrane affinity of active site modified APC may result in a more therapeutically effective polypeptide.

15 The vitamin k-dependent polypeptide may be factor VII or the active form of factor VII, factor VIIa. Native or naturally-occurring factor VII polypeptide has low affinity for membranes. Amino acid sequences of the wild-type human (hVII) and bovine (bVII) factor VII GLA domain are shown in Table 2.

TABLE 2

20 hVII: ANA-FLXXLRP<sub>11</sub>GSLRXCKXX<sub>21</sub>QCSFXXARXI<sub>31</sub>FKDAXRTKLF<sub>41</sub>WISY (SEQ ID NO:3)

bVII: ANG-FLXXLRP<sub>11</sub>GSLRXCRXX<sub>21</sub>LCSFXXAHXI<sub>31</sub>FRNXXRTRQF<sub>41</sub>WVSY (SEQ ID NO:4)

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The GLA domain of factor VII or VIIa can contain a substitution, for example, at amino acid 11, 12, 29, or 33. The modified GLA domain of factor VII or factor VIIa may include, for example, a glutamic acid, a glutamine, an asparagine, or an aspartic acid residue at amino

acid 11, a phenylalanine or a glutamic acid residue at amino acid 29, or an aspartic acid or a glutamic acid residue at amino acid 33. The modified GLA domain can include combinations of such substitutions at amino acid residues 11 and 29, at residues 11 and 33, at residues 11, 29 and 33, or at residues 29 and 33. For example, the GLA domain of factor VII 5 or factor VIIa may include a glutamine residue at amino acid 11 and a glutamic acid residue at amino acid 33, or a glutamine residue at amino acid 11 and a phenylalanine residue at amino acid 29. Vitamin k-dependent polypeptide modified in this manner has a much higher affinity for membranes than the native or wild type polypeptide. It also has a much higher activity in autoactivation, in factor Xa generation and in several blood clotting assays.

10 Activity is particularly enhanced at marginal coagulation conditions, such as low levels of tissue factor and/or phospholipid. For example, modified factor VII is about 4 times as effective as native VIIa at optimum thromboplastin levels, but is about 20-fold as effective at 1% of optimum thromboplastin levels. Marginal pro-coagulation signals are probably most predominant *in vivo*. Presently available clotting assays that use optimum levels of 15 thromboplastin cannot detect clotting time differences between normal plasma and those from hemophilia patients. Clotting differences between such samples are only detected when non-optimal levels of thromboplastin or dilute thromboplastin are used in clotting assays.

Another example of a vitamin k-dependent polypeptide is active-site modified Factor VIIa. The active site of factor VIIa may be modified chemically, for example, by 20 DEGR or by site-directed mutagenesis of the active site. DEGR-modified factor VII is an effective inhibitor of coagulation by several routes of administration. Arnljots, B. et al., 1997, J. Vasc. Surg., 25:341-346. Modifications of the GLA domain may make active-site modified Factor VIIa more efficacious due to higher membrane affinity. The modified GLA domain of active-site modified Factor VIIa may include substitutions as described above for 25 Factor VII. For example, a glutamine residue at amino acid 11 and a glutamic acid residue at amino acid 33.

The vitamin K-dependent polypeptide may also be Factor IX or the active form of Factor IX, Factor IXa. As with active site-modified factor VIIa, active site modified IXa and Xa may be inhibitors of coagulation. Amino acid sequences of the wild-type human (hIX)

and bovine (bIX) factor IX GLA domain are shown in Table 3. Suitable substitutions for factor IX are described above. For example, substitutions can include an asparagine, an aspartic acid or glutamic acid residue at amino acid 11, a phenylalanine or glutamic acid residue at amino acid 29, a glutamine or aspartic acid residue at amino acid 33, or an aspartic 5 acid residue at amino acid 34.

TABLE 3

**bIX: YNSGKLXXFVQ<sub>11</sub>GNLXRXCMXX<sub>21</sub>KCSFXXARXV<sub>31</sub>FXNTXRTTXF<sub>41</sub>WKQY (SEQ ID NO:5)**

10 **bIX: YNSGKLXXFVQ<sub>11</sub>GNLXRXCMXX<sub>21</sub>KCSFXXARXV<sub>31</sub>FXNTXKRTTXF<sub>41</sub>WKQY (SEQ ID NO:6)**

In another aspect, the invention features a mammalian host cell including a vitamin k-dependent polypeptide having a modified GLA domain that enhances membrane binding affinity of the polypeptide relative to a corresponding native vitamin k-dependent 15 polypeptide. The modified GLA domain includes at least one amino acid substitution as discussed above. The mammalian host cell may include, for example, modified factor VII or modified factor VIIa, a discussed above. The GLA domain of modified factor VII or modified factor VIIa may contain, for example, an amino acid substitution at amino acid 11 and at amino acid 33. Preferably, the amino acid substitution includes a glutamine residue at 20 amino acid 11 and a glutamic acid residue at amino acid 33.

Suitable mammalian host cells are able to modify vitamin k-dependent polypeptide glutamate residues to  $\gamma$ -carboxyglutamate. Mammalian cells derived from kidney and liver are especially useful as host cells.

25 *Nucleic Acids encoding modified vitamin K-dependent polypeptides*

Isolated nucleic acid molecules encoding modified vitamin K-dependent polypeptides of the invention can be produced by standard techniques. As used herein, "isolated" refers to a sequence corresponding to part or all of a gene encoding a modified vitamin K-dependent polypeptide, but free of sequences that normally flank one or both sides

of the wild-type gene in a mammalian genome. An isolated polynucleotide can be, for example, a recombinant DNA molecule, provided one of the nucleic acid sequences normally found immediately flanking that recombinant DNA molecule in a naturally-occurring genome is removed or absent. Thus, isolated polynucleotides include, without limitation, a

5 recombinant DNA that exists as a separate molecule (e.g., a cDNA or genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences as well as recombinant DNA that is incorporated into a vector, an autonomously replicating plasmid, a virus (e.g., a retrovirus, adenovirus, or herpes virus), or into the genomic DNA of a prokaryote or eukaryote. In addition, an isolated polynucleotide can

10 include a recombinant DNA molecule that is part of a hybrid or fusion polynucleotide.

It will be apparent to those of skill in the art that a polynucleotide existing among hundreds to millions of other polynucleotides within, for example, cDNA or genomic libraries, or gel slices containing a genomic DNA restriction digest is not to be considered an isolated polynucleotide.

15 Isolated nucleic acid molecules are at least about 14 nucleotides in length. For example, the nucleic acid molecule can be about 14 to 20, 20-50, 50-100, or greater than 150 nucleotides in length. In some embodiments, the isolated nucleic acid molecules encode a full-length modified vitamin K-dependent polypeptide. Nucleic acid molecules can be DNA or RNA, linear or circular, and in sense or antisense orientation.

20 Specific point changes can be introduced into the nucleic acid sequence encoding wild-type vitamin K-dependent polypeptides by, for example, oligonucleotide-directed mutagenesis. In this method, a desired change is incorporated into an oligonucleotide, which then is hybridized to the wild-type nucleic acid. The oligonucleotide is extended with a DNA polymerase, creating a heteroduplex that contains a mismatch at the introduced point change, 25 and a single-stranded nick at the 5' end, which is sealed by a DNA ligase. The mismatch is repaired upon transformation of *E. coli*, and the gene encoding the modified vitamin K-dependent polypeptide can be re-isolated from *E. coli*. Kits for introducing site-directed mutations can be purchased commercially. For example, Muta-Gene® *in-vitro* mutagenesis kits can be purchased from Bio-Rad Laboratories, Inc. (Hercules, CA).

Polymerase chain reaction (PCR) techniques also can be used to introduce mutations. See, for example, Vallette et al., Nucleic Acids Res., 1989, 17(2):723-733. PCR refers to a procedure or technique in which target nucleic acids are amplified. Sequence information from the ends of the region of interest or beyond typically is employed to design 5 oligonucleotide primers that are identical in sequence to opposite strands of the template to be amplified, whereas for introduction of mutations, oligonucleotides that incorporate the desired change are used to amplify the nucleic acid sequence of interest. PCR can be used to amplify specific sequences from DNA as well as RNA, including sequences from total genomic DNA or total cellular RNA. Primers are typically 14 to 40 nucleotides in length, 10 but can range from 10 nucleotides to hundreds of nucleotides in length. General PCR techniques are described, for example in PCR Primer: A Laboratory Manual, Ed. by Dieffenbach, C. and Dveksler, G., Cold Spring Harbor Laboratory Press, 1995.

Nucleic acids encoding modified vitamin K-dependent polypeptides also can be produced by chemical synthesis, either as a single nucleic acid molecule or as a series of 15 oligonucleotides. For example, one or more pairs of long oligonucleotides (e.g., >100 nucleotides) can be synthesized that contain the desired sequence, with each pair containing a short segment of complementarity (e.g., about 15 nucleotides) such that a duplex is formed when the oligonucleotide pair is annealed. DNA polymerase is used to extend the oligonucleotides, resulting in a double-stranded nucleic acid molecule per oligonucleotide 20 pair, which then can be ligated into a vector.

*Production of modified vitamin K-dependent polypeptides*

Modified vitamin K-dependent polypeptides of the invention can be produced by ligating a nucleic acid sequence encoding the polypeptide into a nucleic acid construct such as an expression vector, and transforming a bacterial or eukaryotic host cell with the 25 expression vector. In general, nucleic acid constructs include a regulatory sequence operably linked to a nucleic acid sequence encoding a vitamin K-dependent polypeptide. Regulatory sequences do not typically encode a gene product, but instead affect the expression of the nucleic acid sequence. As used herein, "operably linked" refers to connection of the regulatory sequences to the nucleic acid sequence in such a way as to permit expression of

the nucleic acid sequence. Regulatory elements can include, for example, promoter sequences, enhancer sequences, response elements, or inducible elements.

In bacterial systems, a strain of *Escherichia coli* such as BL-21 can be used. Suitable *E. coli* vectors include without limitation the pGEX series of vectors that produce 5 fusion proteins with glutathione S-transferase (GST). Transformed *E. coli* are typically grown exponentially, then stimulated with isopropylthiogalactopyranoside (IPTG) prior to harvesting. In general, such fusion proteins are soluble and can be purified easily from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease 10 cleavage sites such that the cloned target gene product can be released from the GST moiety.

In eukaryotic host cells, a number of viral-based expression systems can be utilized to express modified vitamin K-dependent polypeptides. A nucleic acid encoding vitamin K-dependent polypeptide can be cloned into, for example, a baculoviral vector such 15 as pBlueBac (Invitrogen, San Diego, CA) and then used to co- transfect insect cells such as *Spodoptera frugiperda* (Sf9) cells with wild-type DNA from *Autographa californica* multiply enveloped nuclear polyhedrosis virus (AcMNPV). Recombinant viruses producing the modified vitamin K-dependent polypeptides can be identified by standard methodology. Alternatively, a nucleic acid encoding a vitamin K-dependent polypeptide can be introduced 20 into a SV40, retroviral, or vaccinia based viral vector and used to infect host cells.

Mammalian cell lines that stably express modified vitamin K-dependent polypeptides can be produced by using expression vectors with the appropriate control elements and a selectable marker. For example, the eukaryotic expression vector pCDNA.3.1<sup>+</sup> (Invitrogen, San Diego, CA) is suitable for expression of modified vitamin K- 25 dependent polypeptides in, for example, COS cells, HEK293 cells, or baby hamster kidney cells. Following introduction of the expression vector by electroporation, DEAE dextran-, calcium phosphate-, liposome-mediated transfection, or other suitable method, stable cell lines can be selected. Alternatively, transiently transfected cell lines are used to produce modified vitamin K-dependent polypeptides. Modified vitamin K-dependent polypeptides

also can be transcribed and translated *in vitro* using wheat germ extract or rabbit reticulocyte lysate.

Modified vitamin K-dependent polypeptides can be purified from conditioned cell medium by applying the medium to an immunoaffinity column. For example, an antibody 5 having specific binding affinity for Factor VII can be used to purify modified Factor VII.

Alternatively, concanavalin A (Con A) chromatography and anion-exchange chromatography (e.g., DEAE) can be used in conjunction with affinity chromatography to purify factor VII. Calcium dependent or independent monoclonal antibodies that have specific binding affinity for factor VII can be used in the purification of Factor VII.

10 Modified vitamin K-dependent polypeptides such as modified protein C can be purified by anion-exchange chromatography, followed by immunoaffinity chromatography using an antibody having specific binding affinity for protein C.

Modified vitamin K-dependent polypeptides also can be chemically synthesized using standard techniques. See, Muir, T.W. and Kent, S.B., Curr. Opin. Biotechnol., 1993, 15 4(4):420-427, for a review of protein synthesis techniques.

#### *Pharmaceutical Compositions*

The invention also features a pharmaceutical composition including a pharmaceutically acceptable carrier and an amount of a vitamin k-dependent polypeptide 20 effective to inhibit clot formation in a mammal. The vitamin k-dependent polypeptide includes a modified GLA domain with at least one amino acid substitution that enhances membrane binding affinity of the polypeptide relative to a corresponding native vitamin k-dependent polypeptide. Useful modified vitamin k-dependent polypeptides of the pharmaceutical compositions can include, without limitation, protein C or APC, active-site 25 modified APC, active-site modified factor VIIa, active-site modified factor IXa, and active-site modified factor Xa as discussed above.

The concentration of a vitamin k-dependent polypeptide effective to inhibit clot formation in a mammal may vary, depending on a number of factors, including the preferred dosage of the compound to be administered, the chemical characteristics of the compounds

employed, the formulation of the compound excipients and the route of administration. The optimal dosage of a pharmaceutical composition to be administered may also depend on such variables as the overall health status of the particular patient and the relative biological efficacy of the compound selected. These pharmaceutical compositions may be used to

5 regulate coagulation *in vivo*. For example, the compositions may be used generally for the treatment of thrombosis. Altering only a few amino acid residues of the polypeptide as described above, generally does not significantly affect the antigenicity of the mutant polypeptides.

Vitamin k-dependent polypeptides that include modified GLA domains may be

10 formulated into pharmaceutical compositions by admixture with pharmaceutically acceptable non-toxic excipients or carriers. Such compounds and compositions may be prepared for parenteral administration, particularly in the form of liquid solutions or suspensions in aqueous physiological buffer solutions; for oral administration, particularly in the form of tablets or capsules; or for intranasal administration, particularly in the form of powders, nasal

15 drops, or aerosols. Compositions for other routes of administration may be prepared as desired using standard methods.

Formulations for parenteral administration may contain as common excipients sterile water or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes, and the like. In particular, biocompatible, biodegradable

20 lactide polymer, lactide/glycolide copolymer, or polyoxethylene-polyoxypropylene copolymers are examples of excipients for controlling the release of a compound of the invention *in vivo*. Other suitable parenteral delivery systems include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes.

Formulations for inhalation administration may contain excipients such as lactose, if desired.

25 Inhalation formulations may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or they may be oily solutions for administration in the form of nasal drops. If desired, the compounds can be formulated as gels to be applied intranasally. Formulations for parenteral administration may also include glycocholate for buccal administration

In an alternative embodiment, the invention also features a pharmaceutical composition including a pharmaceutically acceptable carrier and an amount of a vitamin k-dependent polypeptide effective to increase clot formation in a mammal. The vitamin k-dependent polypeptide includes a modified GLA domain with at least one amino acid

5 substitution that enhances membrane binding affinity of the polypeptide relative to a corresponding native vitamin k-dependent polypeptide. These pharmaceutical compositions may be useful for the treatment of clotting disorders such as hemophilia A, hemophilia B and liver disease.

In this embodiment, useful vitamin k-dependent polypeptides of the

10 pharmaceutical compositions can include, without limitations, Factor VII or the active form of Factor VII, Factor VIIa. The modified GLA domain of Factor VII or Factor VIIa may include substitutions at amino acid 11 and amino acid 33, for example, a glutamine residue at amino acid 11 and a glutamic acid residue at amino acid 33. The pharmaceutical composition may further comprise soluble tissue factor. Factor VII is especially critical to

15 blood coagulation because of its location at the initiation of the clotting cascade, and its ability to activate two proteins, factors IX and X. Direct activation of factor X by factor VIIa is important for possible treatment of the major forms of hemophilia, types A and B, since the steps involving factors IX and VIII are bypassed entirely. Administration of factor VII to patients has been found to be efficacious for treatment of some forms of hemophilia.

20 Improvement of the membrane affinity of factor VII or VIIa by modification of the GLA domain provides the potential to make the polypeptide more responsive to many coagulation conditions, to lower the dosages of VII/VIIa needed, to extend the intervals at which factor VII/VIIa must be administered, and to provide additional qualitative changes that result in more effective treatment. Overall, improvement of the membrane contact site of factor VII

25 may increase both its activation rate as well as improve the activity of factor VIIa on factor X or IX. These steps may have a multiplicative effect on overall blood clotting rates *in vivo*, resulting in a very potent factor VIIa for superior treatment of several blood clotting disorders.

Other useful vitamin k-dependent polypeptides for increasing clot formation include Factor IX and Factor IXa.

In another aspect, methods for decreasing clot formation in a mammal are described. The method includes administering an amount of vitamin k-dependent 5 polypeptide effective to decrease clot formation in the mammal. The vitamin k-dependent polypeptide includes a modified GLA domain that enhances membrane binding affinity of the polypeptide relative to a corresponding native vitamin k-dependent polypeptide. The modified GLA domain includes at least one amino acid substitution. Modified protein C or APC or modified active-site blocked factors VIIa, IXa, Xa and APC may be used for this 10 method.

In another aspect, the invention also features methods for increasing clot formation in a mammal that includes administering an amount of vitamin k-dependent polypeptide effective to increase clot formation in the mammal. The vitamin k-dependent polypeptide includes a modified GLA domain that enhances membrane binding affinity of 15 the polypeptide relative to a corresponding native vitamin k-dependent polypeptide. The modified GLA domain includes at least one amino acid substitution. Modified factor VII or VIIa and modified factor IX or IXa may be used in this method.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

20

### Examples

Example 1 - Factor VII with Enhanced Membrane Affinity and Activity: It has been found that the membrane binding affinity of human blood clotting factor VII can be increased by site-directed mutagenesis. The properties of a P11Q,K33E mutant (referred to 25 herein as Factor VIIQ11E33 or mutant factor VII) have been characterized. Membrane affinity was increased over wild type protein by about 20-fold. Autoactivation by the mutant affinity was increased by at least 100-fold over that of wild type factor VII. The activated form of VIIQ11E33 (referred to as VIIaQ11E33) displayed about 10-fold higher activity toward factor X. The coagulation activity of VIIaQ11E33 with soluble tissue factor in normal

plasma was about 10-fold higher than that of wild type VIIa. Coagulation activity of the zymogen, VIIQ11E33, with normal tissue factor (supplied as a 1:100 dilution of thromboplastin-HS), was 20-fold higher than wild type Factor VII. The degree to which activity was enhanced was dependent on conditions, with VIIQ11E33 being especially active  
5 under conditions of low coagulation stimuli.

In general, protein concentrations were determined by the Bradford assay using bovine serum albumin as the standard. Bradford, M.M., 1976, Analyt. Biochem. 248-254. Molar concentrations were obtained from the molecular weights of 50,000 for factor VII and 55,000 for factor X. Unless indicated, all activity measurements were conducted in standard  
10 buffer (0.05 M Tris, pH 7.5, 100 mM NaCl).

Production of Mutant Factor VII: Mutant factor VII was generated from wild type factor VII cDNA (GenBank Accession number M13232, NID g182799). Petersen et al., 1990, Biochemistry 29:3451-3457. The P11Q mutation (change of amino acid 11 from a proline residue to a glutamine residue) and the K33E mutation (change of amino acid 33  
15 from a lysine residue to a glutamic acid residue) were introduced into the wild type factor VII cDNA by a polymerase chain reaction strategy essentially as described by Vallette et al., 1989, Nucleic Acids Res. 17:723-733. During this process, a mutation-diagnostic *Xma*III restriction enzyme site was eliminated. Four PCR primers were designed to prime synthesis of two mutant fragments of M13232, one from *Mlu*I to *Bgl*II, positions 221 to 301, and the  
20 other from *Bgl*II to *Sst*II, positions 302 to 787. These primers were used under standard PCR cycling conditions (GENEAMP, Perkin Elmer) to prime fragment synthesis using 1 ng of the wild-type factor VII cDNA as template. The resulting fragments were gel purified and digested with *Mlu*I and *Bgl*II or *Bgl*II and *Sst*II. The two purified fragments were then ligated into the factor VIII cDNA in the expression vector Zem219b from which the  
25 corresponding wild-type sequence had been removed as a *Mlu*I-*Sst*II fragment. Petersen et al., 1990 *supra*. The mutated fragments were sequenced in their entirety to confirm the P11Q and K33E substitutions, as well as to eliminate the possibility of other PCR-induced sequence changes.

Transfection, Selection and Purification: Baby hamster kidney (BHK) cells were grown in Dubeccos modified Eagles medium supplemented with 10% fetal calf serum and penicillin-streptomycin. Subconfluent cells were transfected with the factor VII expression plasmid using lipofectAMINE (Gibco BRL) according to the manufacturers

5 recommendations. Two days post-transfection, cells were trypsinized and diluted to selective medium containing 1  $\mu$ M methotrexate (MTX). Stably-transfected BHK cells were subsequently cultured in serum-free Dubeccos modified Eagles medium supplemented with penicillin-streptomycin, 5  $\mu$ g/mL vitamin K<sub>1</sub> and 1  $\mu$ M MTX, and conditioned medium was collected. The conditioned medium was applied twice to an immunoaffinity column

10 composed of a calcium-dependent monoclonal antibody (CaFVII22) coupled to Affi-Gel 10. Nakagaki *et al.*, 1991, Biochemistry, 30:10819-10824. The final purified Factor VIIQ11E33 ran as a single band on SDS polyacrylamide gel electrophoresis, with no evidence of factor VIIa in the preparation. The pure VII(P11Q,K33E) mutant showed 1400-2800 factor VII units/mg.

15 Activation of Factor VII: Activated Factor VIIaQ11E33 was formed by bovine factor Xa cleavage of VIIQ11E33 (1:100 weight ratio, incubation for 1 hr at 37°C). Alternatively, Factor VIIaQ11E33 was obtained by autoactivation (37°C, 20 min) in a mixture containing 7  $\mu$ M VIIQ11E33, 0.7  $\mu$ M sTF and phospholipid (phosphatidylserine / phosphatidylcholine (PS/PC), 25/75, 0.1 g/g protein).

20 Wild-type factor VIIa was a homogeneous, recombinant protein (NOVO Nordisk). Two preparations consisted of a commercial, lyophilized product and non-lyophilized product. The latter protein was further purified on FPLC mono-Q and showed a specific activity of 80,000 units/mg, calibrated with a George King NPP standard.

Enhanced membrane interaction by Factor VIIQ11E33: Phospholipid

25 preparation, assay and measurement of protein-membrane binding was conducted by the method described by Nelsestuen and Lim, 1977, Biochemistry, 30:10819-10824. Large unilamellar vesicles (LUVs) and small unilamellar vesicles (SUVs) were prepared by methods described previously. Hope, M.J., et al., Biochem. Biophys. Acta., 812:55-65; Huang, C., 1969, Biochemistry, 8:344-352. Highly pure phosphatidylserine (bovine brain)

and egg phosphatidylcholine (Sigma Chemical Co.) were mixed in chloroform. The solvent was removed by a stream of nitrogen gas. The dried phospholipids were suspended in buffer. SUVs were formed by sonication and gel filtration while LUVs were formed by freeze-thaw and extrusion. Phospholipid concentrations were determined by organic phosphate assay

5 assuming a phosphorous:phospholipid weight ratio of 25.

SUVS of either PS/PC (25/75) or PS/PC (10/90) were prepared. Protein was added to phospholipid at the weight ratios shown in Figure 1. Protein-membrane binding was assayed by light scattering at 90° by the method of Nelsestuen and Lim, 1977 *supra*.

Briefly, the light scattering intensity of phospholipid vesicles alone ( $I_1$ ) and after addition of 10 protein ( $I_2$ ) were measured and corrected for background from buffer and unbound protein.

The molecular weight ratio of the protein-vesicle complex ( $M_2$ ) to that of the vesicles alone ( $M_1$ ), can be estimated from the relationship in equation 1, where  $\delta n/\delta c$  is the refractive index of the respective species.

15  $I_2/I_1 = (M_2/M_1)^2 (\delta n/\delta c_2 / \delta n/\delta c_1)^2$  (eq. 1)

If phospholipid and protein concentrations are known, the concentration of bound [ $P^*PL$ ] and free protein [P] can be estimated. These values, together with the maximum protein binding capacity [ $P^*PL_{max}$ ] of the vesicles (assumed to be 1.0 g/g for all proteins) can be used to

20 obtain the equilibrium constant for protein-membrane interaction by the relationship in equation 2, where all concentrations are expressed as molar protein or protein binding sites.

$$K_D = [P][P^*PL_{max} - P^*PL]/[P^*PL] \quad (\text{eq. 2})$$

25 Binding was assessed at 5 mM calcium and is expressed as the ratio,  $M_2/M_1$ .

Figure 1 shows the binding of wild type VIIa (open circles) and factor VIIQ11E33 (filled circles) to membranes of either PS/PC=25/75, 25 µg/ml (Figure 1A) or PS/PC=10/90, 25 µg/ml (Figure 1B). VIIQ11E33 had much higher affinity than wild type protein. Binding to PS/PC (25/75) was at the quantitative level so that [Protein<sub>free</sub>] was essentially zero.

30 Consequently, Kd values could not be estimated from this data. Membrane binding of

bovine factor X (filled triangles) is shown in Figure 1 as a reference. Bovine factor X is one of the highest affinity proteins in this family, giving a  $K_d$  for PS/PC (20/80) at 2 mM calcium of 40 nM. McDonald et al., 1997, *Biochemistry*, 36:5120-5127. The  $K_d$  for bovine factor X, obtained from the result at a protein/phospholipid ratio of 0.55 (Figure 1), was 0.025  $\mu$ M.

5 Binding of wild-type and mutant Factor VII to membranes of PS/PC (10/90) was also determined (Figure 1B). The VIIQ11E33 bound at less than the quantitative level, which allowed a binding constant to be estimated from the relationship in equation 3.

$$K_d = [Protein_{free}][Binding\ sites_{free}]/[Protein_{bound}] \quad (eq. 3)$$

10

[Binding sites<sub>free</sub>] were estimated from equation 4, assuming a maximum M2/M1 of 1.0 (i. e., [Binding sites<sub>total</sub>]=[Phospholipid weight conc./Protein<sub>MW</sub>]). This is a common value observed for several proteins of this family. See McDonald et al., 1997, *supra*.

15

$$[Binding\ sites_{free}] = [Binding\ sites_{total}] - [Protein_{bound}] \quad (eq. 4)$$

20

Using these assumptions and the data at a protein to phospholipid ratio of 0.37,  $K_d$  values were 0.7  $\mu$ M for bovine factor X, 5.5  $\mu$ M for wild type factor VII and 0.23  $\mu$ M for VIIQ11E33. Thus, it was clear that factor VIIQ11E33 was greatly improved in membrane binding affinity over wild type factor VII and had one of the highest membrane-binding affinities among the vitamin K-dependent proteins.

25

Enhanced activation of factor VIIQ11E33: The first step in coagulation involves the activation of factor VII. Autoactivation of VII was conducted in a solution containing 100 nM sTF (highly purified recombinant product from Dr. Walter Kisiel, Fiore et al., 1994, *J. Biol. Chem.*, 269:143-149), 36 nM VIIQ11E33 and PS/PC (25/75, 22  $\mu$ g/mL). Activity of VIIaQ11E33 was estimated at various time intervals by addition of 0.15 mm substrate S-2288 (Kabi) and assessing the rate of p-nitrophenylphosphate product release by absorbance change at 405 nm. Initial activity of the VIIQ11E33 preparation was less than 4% that of fully active VIIaQ11E33.

VIIQ11E33 was found to be a much better substrate for activation than wild-type factor VII. Figure 2 shows autoactivation of factor VIIQ11E33. The data were analyzed by the relationship in equation 5 (equation 7 of Fiore et al., 1994, *supra*).

5  $\ln[\text{VIIa}]_t = \ln[\text{VIIa}]_0 + \text{kcat} \cdot y \cdot t$  (eq. 5)

$\ln[\text{VIIa}]_t$  is the factor VIIa concentration at time  $t$ ,  $\text{kcat}$  is the catalytic rate constant for factor VIIa acting on VII and  $y$  is the fractional saturation of VIIa sites. For wild-type factor VIIa, this relationship and  $1 \mu\text{M}$  sTF gave a  $\text{kcat}$  of  $0.0045/\text{s}$  and a  $\text{kcat}/\text{Km}$  ratio of  $7 \cdot 10^3 \text{ M}^{-1}\text{s}^{-1}$ .

10 See, Fiore et al., 1994, *supra*. For the VIIQ11E33 enzyme, autoactivation was rapid (Figure 2) and it was only possible to estimate a lower limit for  $\text{kcat}$ . This was obtained from the VIIa doubling time of about 25 seconds ( $\text{kcat} = (\ln 2)/t_{1/2}$ ). The resulting value ( $\text{kcat}_{\min} = 0.03/\text{s}$ ), along with the substrate concentration of this reaction ( $3.6 \cdot 10^{-8} \text{ M}$ ) and the assumption that  $y=1.0$ , gave a value for  $\text{kcat}/[\text{S}] = 8 \cdot 10^5 \text{ M}^{-1}\text{s}^{-1}$ . This should be far below the true  $\text{kcat}/\text{Km}$  for 15 VIIaQ11E33, but was about 100-times greater than the value of  $\text{kcat}/\text{Km}$  for wild type factor VIIa/sTF estimated by Fiore et al., 1994, *supra*. Thus, the combination of VIIaQ11E33 enzyme and Factor VIIQ11E33 substrate was superior to wild type proteins in the activation step of coagulation. This suggested that VIIQ11E33 was superior to wild type enzyme when coagulation conditions were minimal.

20 Enhanced activity of VIIaQ11E33: Once generated, factor VIIa activates either factor X or factor IX. Activation of bovine factor X ( $0.1 \mu\text{M}$ ) by factor VIIa was carried out in  $50 \text{ mM}$  Tris-HCl buffer, pH 7.5 containing  $100 \text{ mM}$  NaCl,  $5 \text{ mM}$  calcium, various amounts of phospholipid (PS/PC, 25/75) and  $1 \text{ mg/mL}$  bovine serum albumin at  $22.5^\circ\text{C}$ . Factor VIIa (0.06 nM of VIIaQ11E33 or 0.6 nM wild type VIIa) was added at zero time and Xa activity 25 at 1, 3 and 5 minute time points was determined. Aliquots of the reaction mixture ( $0.2 \text{ mL}$ ) were mixed with buffer ( $0.2 \text{ mL}$ ) containing  $10 \text{ mM}$  EDTA and  $0.4 \text{ mM}$  S-2222 (Kabi), a chromogenic substrate for factor Xa. Absorbance change at  $405 \text{ nm}$  was determined in a Beckman DU8 spectrophotometer. The amount of factor Xa generated was calculated from the extinction coefficient ( $1 \cdot 10^4 \text{ M}^{-1}\text{cm}^{-1}$ ) of the p-nitrophenylphosphate reaction product and

a velocity of 33/sec for substrate hydrolysis by purified bovine Xa under the conditions of this assay.

Figure 3 compares the ability of wild type factor VIIa (open circles) and VIIaQ11E33 (closed circles) to activate factor X in a purified system. Again, VIIaQ11E33 5 was far superior to wild type factor VIIa in this reaction. The difference was greatest at low phospholipid concentrations and diminished to 2-fold at 200  $\mu$ g phospholipid per mL. This was expected from the fact that high membrane concentrations cause a greater portion of wild type VIIa to bind to the membrane. Once again, the increased function of VIIaQ11E33 was greatest under conditions of low phospholipid exposure.

10 Superior coagulation of VIIaQ11E33: Blood clotting assays were conducted at 37°C using the hand tilt method to detect clot formation. Human plasma (0.1 mL) was allowed to equilibrate at 37°C for 1 minute. Various reagents were added in a volume of 0.1 mL of standard buffer. Soluble tissue factor (50 nM) and phospholipid (PS/PC, 10/90, 75  $\mu$ g/mL) were added to the plasma, along with the factor VIIa concentration shown in Figure 15 4 (0.1 - 32 nM). Finally, 0.1 mL of 25 mM CaCl<sub>2</sub> was added to start the reaction. Time to form a clot was measured. In most cases, the average and standard deviations of replicate samples was reported.

Figure 4 shows the coagulation times of wild type VIIa versus VIIaQ11E33 in 20 normal human plasma. Coagulation was supported by sTF and added phospholipid vesicles. Endogenous wild type factor VII is approximately 10 nM in concentration, and had virtually no impact on coagulation times. The background coagulation was 120 seconds, with or without sTF. Factor VIIaQ11E33 showed approximately 8-fold higher activity than the wild type VIIa under these assay conditions. Similar results were obtained with factor VIII-deficient plasma, suggesting that the major pathway for blood clotting in this system 25 involved direct activation of factor X by factor VIIa. Overall, factor VIIaQ11E33 was superior to wild type VIIa in procoagulant activity supported by membrane vesicles and soluble tissue factor. Wild type zymogen had virtually no activity under these conditions, as indicated by similar background clotting times of 2 minutes, whether or not sTF was added.

Procoagulant activity with normal tissue factor: Activity of VIIa and/or VIIQ11E33 with sTF was measured in normal human plasma. Endogenous factor VII appeared to have no impact on coagulation time in this assay; the background clotting time was 2 minutes for plasma with or without soluble tissue factor. Soluble tissue factor (50 nM final concentration) and VIIa were added to the plasma before the calcium solution. 5 Coagulation time was assessed for samples containing various levels of VIIa or VIIaQ11E33. Two human plasma preparations were tested, normal and factor VIII-deficient.

Coagulation supported by normal tissue factor was assayed with standard rabbit brain thromboplastin-HS (HS=high sensitivity) containing calcium (Sigma Chemical Co.). 10 This mixture contains both phospholipids and membrane-bound tissue factor. Rabbit brain thromboplastin-HS was diluted 1:100 in buffer and used in the assay of VII (added in the form of normal human plasma, which contains 10 nM factor VII) and VIIQ11E33 (added as the pure protein). The thromboplastin (0.2 mL) was added to plasma (0.1 mL) to start the reaction and the time required to form a blood clot was measured. Assays were also 15 conducted with full strength thromboplastin, as described by the manufacturer.

At optimum levels of human thromboplastin, wild type VII showed a normal level of activity, about 1500 units per mg. This is approximately 25-fold less than the activity of wild type factor VIIa (80,000 units per mg). The VIIQ11E33 gave approximately 1500-3000 units per mg under the standard assay conditions, only 2-fold greater than wild type VII. 20

The difference between wild type VII and VIIQ11E33 was much greater when the coagulation conditions were sub-optimal. Figure 5 shows the clotting times and zymogen concentrations in assays that contained 0.01-times the normal thromboplastin level. Under these conditions, VIIQ11E33 was approximately 20-fold more active than wild type factor VII. Thus, greater efficacy of the VIIQ11E33 mutant was especially evident when 25 coagulation conditions were limited, which is relevant to many situations *in vivo*.

Anticoagulant Activities of DEGR-VIIaQ11E33: Standard coagulation assays were performed with normal human serum and human thromboplastin that was diluted 1:10 with buffer. The active site of factor VIIaQ11E33 was modified by DEGR as described by Sorenson, B.B. et al., 1997, *supra*. Figure 6 shows the clotting time of DEGR-VIIaQ11E33

(0-4 nm) incubated with thromboplastin, in calcium buffer, for 15 seconds before addition of the plasma. The time to form a clot was determined with the hand tilt method. Clotting time was approximately 45 seconds with about 1 nm of DEGR-VIIaQ11E33.

5        Example 2 - Purification of Factor VII: Factor VII (wild-type or mutant) was purified by Conconavalin A (Con A), DEAE, and affinity chromatography. Crude media of transfected 293 cells were incubated with Con A resin (Pharmacia) for four hours at 4°C. The resin then was washed with a solution containing 50 mM Tris, pH 7.5, 10 mM benzamidine, 1 mM CaCl<sub>2</sub>, and 1 mM MgCl<sub>2</sub>, and factor VII was eluted with 0.2 M D-methyl 10 mannoside, 0.5 M NaCl in 50 mM Tris buffer, pH 7.5. Factor VII was dialyzed against 50 mM Tris, pH 8.0, 50 mM NaCl, 10 mM benzamidine, and 25 mM D-methyl mannoside overnight.

15        Dialyzed factor VII then was incubated with DEAE resin (Pharmacia) for one hour and the mixture was packed into a column. The DEAE column was washed with 50 mM Tris, pH 8.0, 10 mM Benzamidine, and 50 mM NaCl, and factor VII was eluted with a gradient from 50 mM to 500 mM NaCl in 50 mM Tris buffer, pH 8.0 at a flow rate of 2 mL/min. Fractions containing factor VII activity were pooled and dialyzed against 50 mM Tris, pH 8.0, 50 mM NaCl, and 5 mM CaCl<sub>2</sub> overnight. The Con A and DEAE partially purified factor VII was activated by bovine factor Xa (weight ratio 1:10, Enzyme Research 20 Laboratory) at 37°C for one hour.

25        Activated factor VII was purified further by affinity chromatography. A calcium-independent monoclonal antibody for factor VII (Sigma) was coupled to affigel-10 (Bio-Rad Laboratory) as described by Broze et al., J. Clin. Invest., 1985, 76:937-946, and was incubated with the affinity column overnight at 4°C. The column was washed with 50 mM Tris, pH 7.5, 0.1 M NaCl, and factor VIIa was eluted with 50 mM Tris, pH 7.5, 3 M NaSCN at a flow rate of 0.2 mL/min. The eluted fractions were immediately diluted five fold into 50 mM Tris, pH 7.5, 0.1 M NaCl. Fractions containing factor VIIa activity were pooled, concentrated, and dialyzed against 50 mM Tris, pH 7.5, 0.1 M NaCl overnight.

The protein concentration of factor VIIa was determined with a Bio-Rad protein assay kit, using BSA as the standard. The purity of factor VIIa was assayed by Coomassie gel and Western Blotting under reduced and denatured conditions. Proteolytic activity of factor VIIa was measured using a synthetic peptide substrate spectrozyme-FVIIa (American 5 Diagnostics) in the presence of thromboplastin (Sigma). Purified factor VIIa was stored in 0.1 mg/ml BSA, 0.1 M NaCl, 50 mM Tris, pH 7.5 at -80°C.

Procoagulant effectiveness of factor VIIa mutants was assessed using standard *in vitro* clotting assays (and modifications thereof); specifically, the prothrombin time (PT) assay and the activated partial thromboplastin (aPTT) assay. Various concentrations of 10 Factor VIIa mutants were evaluated in pooled normal human donor plasma and in coagulation factor-deficient (Factor VIII, Factor IX, Factor VII) human plasmas (Sigma). Clotting times were determined at 37°C using both a FibroSystem fibrometer (BBL) with a 0.3 mL probe and a Sysmex CA-6000 Automated Coagulation Analyzer (Dade Behring).

Platelet poor plasma (PPP) was prepared from pooled normal human donor blood. 15 Blood (4.5 mLs) was drawn from each healthy donor into citrated (0.5 mLs of 3.2% buffered sodium citrate) Vacutainer tubes. Plasma was obtained after centrifugation at 2,000 g for ten minutes and was kept on ice prior to use. Purchased factor-deficient plasmas were reconstituted according to the manufacturer's instructions. Serial dilutions from each stock of Factor VIIa mutant were all prepared in plasma. All plasmas (with or without Factor VIIa 20 mutants) were kept on ice prior to use. In all cases, the time to form a clot was measured. The average and standard deviation of replicate samples was reported.

The prothrombin time (PT) assay was performed in plasmas (with or without serial dilutions of added FVIIa mutants) using either of the following PT reagents: Thromboplastin C-Plus (Dade), Innovin (Dade), Thromboplastin With Calcium (Sigma), or 25 Thromboplastin HS With Calcium (Sigma). Assays were conducted according to manufacturer's instructions. In addition to using PT reagents at the manufactured full-strength concentration, the PT assay also was performed using various dilutions of PT reagent. In all cases, the time to form a clot was measured. The average and standard deviation of replicate samples was reported.

The activated partial thromboplastin (aPTT) assay was performed in plasmas (with or without serial dilutions of added Factor VIIa mutants) using either Actin FS (Dade) or APTT reagent (Sigma). Clotting was initiated using 0.025M CaCl<sub>2</sub> (Dade) for Actin FS (Dade) or 0.02M CaCl<sub>2</sub> (Sigma) for APTT reagent (Sigma). Assays were conducted 5 according to manufacturer's instructions. In addition to using aPTT reagents at the manufactured full-strength concentration, the aPTT assay also was performed using various dilutions of aPTT reagent. In all cases, the time to form a clot was measured. The average and standard deviation of replicate samples was reported.

Clotting assays also were conducted at 37°C in which different concentrations of 10 phospholipid vesicles [at varying ratios of PS/PC or PS/PC/PE (PE=phosphatidylethanolamine)] were added to plasmas (with or without serial dilutions of added FVIIa mutants). Clotting was initiated by the addition of 20 mM CaCl<sub>2</sub>. Various reagents were added in standard buffer. In all cases, the time to form a clot was measured. The average and standard deviation of replicate samples was reported.

15 Specific Factor VIIa clotting activity was assessed in plasmas (with or without added Factor VIIa mutants) using the STACLOT VIIa-rTF kit (Diagnostica Stago), as per the manufacturer's instructions. This kit is based on the quantitative clotting assay for activated FVII (Morrissey et al., 1993, *Blood*, 81(3):734-744). Clotting times were determined using both a FibroSystem fibrometer (BBL) with a 0.3 mL probe and a Sysmex CA-6000 20 Automated Coagulation Analyzer (Dade Behring).

Example 3 - Circulatory Time of Factor VIIQ11E33 in the Rat: Two anesthetized (sodium nembutal) Sprague Dawley rats (325-350 g) were injected with 36  $\mu$ g of factor VIIQ11E33 at time zero. Injection was through the jugular vein, into which a cannula had 25 been placed. At the times shown in Figure 7, blood was withdrawn from the carotid artery, into which a cannula had been inserted by surgery. The amount of factor VIIQ11E33 in the circulation was estimated from the clotting time of human factor VII-deficient plasma, to which 1  $\mu$ L of a 1:10 dilution of the rat plasma was added. A 1:100 dilution of rabbit brain thromboplastin-HS (Sigma Chemical Co.) was used. Coagulation was assessed by the

manual tube tilt method as described in Example 1. The amount of factor VII activity in the plasma before injection of VIIQ11E33 was determined and was subtracted as a blank. The concentration of factor VIIQ11E33 in the circulation is given as log nM. A sham experiment in which a third animal received the operation and cannulation but no factor VIIQ11E33 was 5 conducted. The amount of factor VII activity in that animal did not change over the time of the experiment (100 minutes). At the end of the experiment, the animals were euthanized by excess sodium nembutol.

The rats appeared normal throughout the experiment with no evidence of coagulation. Therefore, the factor VIIQ11E33 did not cause indiscriminate coagulation, even 10 in the post-operative rat. The circulation life-time of the VIIQ11E33 was normal (Figure 7), with approximately 40% of the protein being cleared in about 60 minutes and an even slower disappearance of the remaining protein. This was similar to the rate of clearance of bovine prothrombin from the rat. Nelsestuen and Suttie, 1971, Biochem. Biophys. Res. Commun., 45:198-203. This is superior to wild-type recombinant factor VIIa which gave a circulation 15 half-time for functional assays of 20-45 minutes. Thomsen, M.K., et al., 1993, Thromb. Haemost., 70:458-464. This indicated that factor VIIQ11E33 was not recognized as an abnormal protein and that it was not rapidly destroyed by coagulation activity. It appeared as a normal protein and should have a standard circulation lifetime in the animal.

20                   Example 4 - Enhancement of the membrane site and activity of protein C: Bovine and human protein C show a high degree of homology in the amino acids of their GLA domains (amino terminal 44 residues), despite about 10-fold higher membrane affinity of the human protein. Bovine protein C contains a proline at position 11 versus a histidine at position 11 of human protein C. The impact of replacing proline-11 in bovine protein C with 25 histidine, and the reverse change in human protein C, was examined. In both cases, the protein containing proline-11 showed lower membrane affinity, about 10-fold for bovine protein C and 5-fold for human protein C. Activated human protein C (hAPC) containing proline at position 11 showed 2.4 to 3.5-fold lower activity than wild type hAPC, depending on the assay used. Bovine APC containing histidine-11 displayed up to 15-fold higher

activity than wild type bAPC. This demonstrated the ability to improve both membrane contact and activity by mutation.

Mutagenesis of Protein C: A full-length human protein C cDNA clone was provided by Dr. Johan Stenflo (Dept. of Clinical Chemistry, University Hospital, Malmö, 5 Sweden). The bovine protein C cDNA clone was provided by Dr. Donald Foster (ZymoGenetics, Inc., USA). The GenBank accession number for the nucleotide sequence of bovine protein C is KO2435, NID g163486 and is K02059, NID g190322 for the nucleotide sequence of human protein C.

Site-directed mutagenesis was performed by a PCR method. For human protein C 10 mutagenesis of histidine-11 to proline, the following oligonucleotides were synthesized: **A**, 5'-AAA TTA ATA CGA CTC ACT ATA GGG AGA CCC AAG CTT-3' (SEQ ID NO:8) (corresponding to nucleotides 860-895 in the vector pRc/CMV) to create a *Hind* III site between pRc/CMV and protein C. **B**, 5'-GCA CTC CCG CTC CAG GCT GCT GGG ACG 15 GAG CTC CTC CAG GAA-3' (SEQ ID NO:8) (corresponding to the amino acid residues 4-17 in human protein C, the 8th residue in this sequence was mutated from that for human protein C to that of bovine protein C, as indicated by the underline).

For bovine protein C mutagenesis of proline-11 to histidine, the following oligonucleotides were synthesized: **A**, (as described above); **C**, 5'-ACG CTC CAC GTT GCC GTG CCG CAG CTC CTC TAG GAA-3' (SEQ ID NO:9) (corresponding to amino acid residues 4-15 in bovine protein C, the 6th amino acid was mutated from that for bovine protein C to that of human protein C as marked with underline); **D**, 5'-TTC CTA GAG GAG CTG CGG CAC GGC AAC GTG GAG CGT-3' (SEQ ID NO:10) (corresponding to amino acid residues 4-15 in bovine protein C, the 7th amino acid was mutated from that for bovine protein C to that of human protein C; mutated nucleotides are underlined); **E**, 5'-GCA TTT 20 AGG TGA CAC TAT AGA ATA GGG CCC TCT AGA-3' (SEQ ID NO:11) (corresponding to nucleotides 984-1019 in the vector pRc/CMV), creating a *Xba* I site between pRc/CMV and protein C).

Both human and bovine protein C cDNAs were cloned into the *Hind* III and *Xba* I sites of the expression vector pRc/CMV. Human protein C cDNA containing the 5' terminus

to amino acid-17 was PCR amplified with intact human protein C cDNA and primers A and B. The volume for the PCR reaction was 100  $\mu$ l and contained 0.25  $\mu$ g of template DNA, 200  $\mu$ M each of the four deoxyribonucleoside triphosphates, 0.5 mM of each primer and 2.5 U of Pwo-DNA polymerase (Boehringer Mannheim) in Tris-HCl buffer (10 mM Tris, 25 mM 5 KCl, 5 mM  $(\text{NH}_4)_2\text{SO}_4$ , and 2 mM MgSO<sub>4</sub>, pH 8.85). Samples were subjected to 30 cycles of PCR consisting of a 2 minute, 94°C denaturation period, a 2 minute, 55°C annealing period, and a 2 minute, 72°C elongation period. After amplification, the DNA was electrophoresed through an 0.8% agarose gel in 40 mM Tris-acetate buffer containing 1 mM EDTA. PCR products were purified with JET Plasmid Miniprep-Kit (Saveen Biotech AB, Sweden).

10 Human protein C cDNA containing respective mutations was cleaved by *Hind III* and *Bsr BI*, and then cloned into pRc/CMV vector that was cleaved by *Hind III/Xba I* and that contained human protein C fragment from *Bsr BI* to the 3' terminus to produce a human protein C full length cDNA with the mutation.

15 Bovine protein C cDNA, containing the 5' terminus through amino acid-11, was PCR amplified with intact human protein C cDNA and primers A and C. Bovine protein C cDNA from amino acid 11 to the 3' terminus was amplified with intact human protein C cDNA and primers D and E. These two cDNA fragments were used as templates to amplify full length bovine protein C cDNA containing mutated amino acids with primers A and E. 20 PCR reaction conditions were identical to those used for hAPC. The bovine protein C cDNA containing the respective mutations was cleaved by *Hind III* and *Bsu 36I*, and the *Hind III/Bsu36I* fragment was cloned into pRc/CMV vector containing intact bovine protein C fragments from the *Bsu 36I* to the 3' terminus to produce full-length bovine protein C cDNA containing the mutation. All mutations were confirmed by DNA sequencing prior to transfection.

25 Cell Culture and Expression: The adenovirus-transfected human kidney cell line 293 was grown in DMEM medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml of penicillin, 100 U/ml streptomycin and 10  $\mu$ g/ml vitamin K<sub>1</sub>. Transfection was performed using the lipofectin method. Felgner, P.L. et al., 1987, Proc. Natl. Acad. Sci. USA, 84:7413-7417. Two  $\mu$ g of DNA was diluted to 0.1 mL with DMEM

containing 2 mM of L-glutamine medium. Ten  $\mu$ L of Lipofectin (1 mg/ml) was added to 100  $\mu$ L of DMEM containing 2 mM L-glutamine medium. DNA and lipofectin were mixed and left at room temperature for 10-15 min. Cell monolayers (25-50% confluence in 5-cm petri-dishes) were washed twice in DMEM with 2 mM L-glutamine medium. The DNA/lipid

5 mixture was diluted to 1.8 mL in DMEM containing 2 mM L-glutamine medium, added to the cells and incubated for 16 hours. The cells were fed with 2 mL of complete medium containing 10% calf serum, left to recover for another 48-72 hours and then trypsinized and seeded into 10-cm dishes with selection medium (DMEM containing 10% serum and 400  $\mu$ g/mL of Geneticin) at 1:5. Yan, S.C.B. et al., 1990, Bio/Technology 655-661. Geneticin-  
10 resistant colonies were obtained after 3-5 weeks of selection. Twenty four colonies from each DNA transfection were picked, grown to confluence and the media screened for protein C expression with a dot-blot assay using monoclonal antibody HPC4 (for human protein C) and monoclonal antibody BPC5 (for bovine protein C). Clones producing high amounts of protein were isolated and grown until confluence in the presence of 10  $\mu$ g/mL of vitamin K<sub>1</sub>.

15 The purification of bovine recombinant protein C and its mutant were based on the method described previously with some modifications. Rezair, A.R., and Esmon, C.T., 1992, J. Biol. Chem., 267:26104-26109. Conditioned serum-free medium from stably transfected cells was centrifuged at 5000 rpm at 4°C for 10 minutes. The supernatant was filtered through 0.45  $\mu$ m of cellulose nitrate membranes (Micro Filtration Systems, Japan).

20 EDTA (5 mM, final concentration) and PPACK (0.2  $\mu$ M, final concentration) were added to the conditioned medium from 293 cells, then passed through a Pharmacia FFQ anion-exchange column at room temperature using Millipore Con Sep LC100 (Millipore, USA). The protein was eluted with a CaCl<sub>2</sub> gradient (starting solution, 20 mM Tris-HCl/150 mM NaCl, pH 7.4; limiting solution, 20 mM Tris-HCl/150 mM NaCl/30 mM CaCl<sub>2</sub>, pH 7.4).

25 After removal of the CaCl<sub>2</sub> by dialysis and Chelex 100 treatment, the protein was reabsorbed to a second FFQ column, then eluted with an NaCl gradient (starting solution 20 mM Tris-HCl/150 mM NaCl, pH 7.4; limiting solution, 20 mM Tris-HCl/500 mM NaCl, pH 7.4). At this point in the purification, wild-type and the mutant recombinant bovine protein C were homogeneous as determined by SDS-PAGE.

The first column used for purification of wild-type and mutant recombinant human protein C was the same as that described for bovine protein C. The chromatographic method described by Rezair and Esmon was employed with some modifications described for the method of protein S purification. Rezair, A.R., and Esmon, C.T., 1992, *supra*; He, Z. et al., 1995, *Eur. J. Biochem.*, 227:433-440. Fractions containing protein C from anion-exchange chromatography were identified by dot-blot. Positive fractions were pooled and applied to an affinity column containing the  $\text{Ca}^{2+}$ -dependent antibody HPC-4. The column was equilibrated with 20 mM Tris-HCl, 150 mM NaCl, pH 7.4, containing 5 mM Benzamidine-HCl and 2 mM  $\text{CaCl}_2$ . After application, the column was washed with the same buffer containing 1 M NaCl. Protein C was then eluted with 20 mM Tris-HCl, 150 mM NaCl and 5 mM EDTA, pH 7.4, containing 5 mM Benzamidine-HCl. After purification, the purity of all human and bovine recombinant protein C preparations was estimated by SDS-PAGE followed by silver staining. Proteins were concentrated using YM 10 filters (Amicon), then dialyzed against buffer (50 mM Tris-HCl and 150 mM NaCl, pH 7.4) for 12 hours and stored at -70°C. The concentrations of proteins were measured by absorbance at 280 nm.

Association of normal and mutant protein C molecules with membranes: LUVs and SUVs were prepared by methods described in Example 1. Light scattering at 90° to the incident light was used to quantitate protein-membrane binding as described above for Factor VII (25  $\mu\text{g}/\text{mL}$  of PS/PC, (25/75) at 5 mM calcium (0.05 M Tris buffer-0.1 M NaCl, pH 7.5).

Bovine protein C containing histidine at position 11 interacted with membranes with about 10-fold higher affinity than wild type protein. When fit to equation 2, the data gave  $K_D$  values of  $930 \pm 80 \text{ nM}$  for protein C-H11 and  $9200 \pm 950 \text{ nM}$  for wild type protein C (Figure 8A). The difference in affinity corresponded to about 1.4 kcal/mol at 25°C. In fact, membrane affinity of bovine protein C-H11 was almost identical to that of native human protein C (660 nM, Figure 8B). This suggested that proline 11 formed a major basis for differences between the membrane binding site of human and bovine proteins.

The reverse substitution, replacement of His-11 of human protein C by proline, decreased membrane affinity (Figure 8B). When fit to equation 2, these data gave  $K_D$  values

of  $660 \pm 90$  nM for wild type human protein C and  $3350 \pm 110$  nM for human protein C-P11. The impact of proline introduction was only slightly less than that of proline in the bovine proteins.

Impact of proline-11 on activity of activated protein C: Activated protein C was 5 generated by thrombin cleavage, using identical conditions for both the wild type and mutant proteins. Approximately  $150 \mu\text{g}$  of the various protein C preparations (1 mg/mL) were mixed with bovine thrombin ( $3 \mu\text{g}$ ) and incubated at  $37^\circ\text{C}$  for 5 hours. The reaction product was diluted to 0.025 M Tris buffer-0.05 M NaCl and applied to a one mL column of SP-Sephadex C-50. The column was washed with one mL of the same buffer and the flow- 10 through was pooled as activated protein C. Approximately 65-80% of the protein applied to the column was recovered. APC activity was determined by proteolysis of S2366 (0.1 mM) at  $25^\circ\text{C}$ . The preparations were compared to standard preparations obtained on larger scale. Standard human APC was provided by Dr. Walter Kisiel. For bovine proteins, the standard was a large-scale preparation of thrombin-activated APC. The activity of bovine APC was 15 consistent for all preparations of normal and mutant proteins ( $\pm 5\%$ ). Two preparations of bovine APC were used for comparisons. Human APC generated from thrombin was 55 to 60% as active as the standard. The concentrations reported in this study were based on activity toward S2366, relative to that of the standard.

Standard APTT test used bovine or human plasma and standard APTT reagent 20 (Sigma Chemical Co.) according to manufacturers instructions. Alternatively, phospholipid was provided in the form of vesicles formed from highly purified phospholipids. In this assay, bovine plasma (0.1 mL) was incubated with either kaolin (0.1 mL of 5 mg/mL in 0.05 M Tris buffer, 0.1 M NaCl, pH 7.5) or ellagic acid (0.1 mM in buffer) for 5 minutes at  $35^\circ\text{C}$ . Coagulation was started by adding 0.1 mL of buffer containing phospholipid and the 25 amounts of APC shown, followed by 0.1 mL of 25 mM calcium chloride. All reagents were in standard buffer containing 0.05 M Tris buffer, 0.1 M NaCl, pH 7.5. An average of a 14-fold higher concentration of wild type bAPC was needed to duplicate the impact of the H11 mutant. Coagulation time at 10 nM bAPC-H11 was greater than 120 minutes. Standard APTT reagent (Sigma Chemical Co.) gave a clotting time of about 61 seconds at  $35^\circ\text{C}$  with

this plasma. Time required to form a clot was recorded by manual technique. The amount of phospholipid was designed to be the limiting component in the assay and to give the clotting times shown. The phospholipids used were SUVs (45  $\mu$ g/0.4 mL in the final assay, PS/PC, 10/90) or LUVs (120  $\mu$ g/0.4 mL in the final assay, PS/PC, 25/75).

5        The anticoagulant activity of activated protein C was tested in several assays. Figure 9 shows the impact on the APTT assay, conducted with limiting phospholipid. Under the conditions of this assay, coagulation times decreased in a nearly linear, inverse relationship with phospholipid concentration. Approximately 14-times as much wild type bovine APC was needed to equal the effect of bovine APC-H11.

10       Parts of the study in Figure 9 were repeated for membranes of PS/PC (25/75, LUV). Again, activity was limited by phospholipid, and its concentration was adjusted to give a control clotting time of 360 seconds (120  $\mu$ g of 25% PS in the 0.4 mL assay). Approximately 15-fold more wild type enzyme was needed to equal the impact of the H11 mutant. Finally, standard APTT reagent (Sigma Chemical Co., standard clotting time  $50 \pm 2$  15 seconds) was used. Approximately  $10.0 \pm 0.7$  nM of wild type enzyme was needed to double the coagulation time to  $102 \pm 5$  seconds. The same impact was produced by  $2.2 \pm 0.1$  nM bovine APC-H11. Phospholipid was not rate limiting in the standard assay so a smaller impact on membrane affinity may be expected.

20       Results for human proteins are shown in Figure 8B. About 2.5 times as much human APC containing proline-11 was required to prolong coagulation to the extent of wild type APC. A lower impact of proline-11 introduction may reflect the smaller differences in membrane affinity of the human proteins (Figure 9B).

25       Inactivation of factor Va: Factor Va inactivation was assayed by the method of Nicolaes et al., 1996, Thrombosis and Haemostasis, 76:404-410. Briefly, for bovine proteins, bovine plasma was diluted 1000-fold by 0.05 M Tris, 0.1 M NaCl, 1 mg/mL bovine serum albumin and 5 mM calcium at pH 7.5. Phospholipid vesicles (5  $\mu$ g/0.24 mL assay) and 5  $\mu$ L of 190 nM thrombin were added to activate factor V. After a 10-minute incubation at 37°C, APC was added and the incubation was continued for 6 minutes. Bovine prothrombin (to 10  $\mu$ M final concentration) and factor Xa (0.3 nM final concentration) were added and the

reaction was incubated for one minute at 37°C. A 20  $\mu$ L sample of this activation reaction was added to 0.38 mL of buffer (0.05 M Tris, 0.1 M NaCl, 5 mM EDTA, pH 7.5) containing S2288 substrate (60  $\mu$ M). The amount of thrombin was determined by the change in absorbance at 405 nM ( $\epsilon=1.0*10^4$  M<sup>-1</sup>s<sup>-1</sup>,  $k_{cat}$  for thrombin=100/s). For human proteins, 5 human protein S-deficient plasma (Biopool Canada, Inc.) was diluted 100-fold, factor Va was activated by human thrombin and the factor Va produced was assayed with the reagents used for the bovine proteins.

Bovine APC-H11 was 9.2-fold more active than wild type (Figure 10A) in inactivating factor Va. As for membrane binding (above), the impact of proline-11 was less 10 with the human proteins, with an average of 2.4-fold difference between the curves drawn for wild type and P-11 mutant (Figure 10B). Similar results were obtained with normal human plasma.

Example 5 - Identification of an archetype membrane affinity for the membrane

15 contact site of Vitamin K-dependent proteins: Comparison of various human and bovine protein C mutants and other vitamin K-dependent polypeptides led to a proposed membrane contact site archetype. The electrostatic archetype consists of an electropositive core on one surface of the protein, created by bound calcium ions, surrounded by a halo of electronegative charge from amino acids of the protein. The closer a member of this protein 20 family approaches this electrostatic pattern, the higher its affinity for membranes.

Phospholipid vesicles, wild type bovine protein C, protein-membrane interaction studies, activation and quantitation of protein C, and activity analysis were as described in Example 4.

Recombinant, mutant protein C was generated by the following procedures. Site- 25 directed mutagenesis was performed by a PCR method. The following oligonucleotides were synthesized: **A**, as described in Example 3; **F**, 5'-GCA TTT AGG TGA CAC TAT AGA ATA GGG CCC TCT AGA -3' (SEQ ID NO:11) (corresponding to nucleotides 984-1019 in the vector pRc/CMV), creating a Xba I site between pRc/CMV and protein C; **G**, 5'-GAA GGC CAT TGT GTC TTC CGT GTC TTC GAA AAT CTC CCG AGC-3' (SEQ ID NO:12)

(corresponding to amino acid residues 40-27 in bovine protein C, the 8th and 9th amino acids were mutated from QN to ED as marked with underline); **H**, 5'-CAG TGT GTC ATC CAC ATC TTC GAA AAT TTC CTT GGC-3' (SEQ ID NO:13) (corresponding to amino acid residues 38-27 in human protein C, the 6th and 7th amino acids in this sequence were 5 mutated from QN to ED as indicated with the underline); **I**, 5'-GCC AAG GAA ATT TTC GAA GAT GTG GAT GAC ACA CTG-3' (SEQ ID NO:14) (corresponding to amino acid residues 27-38 in human protein C, the 6th and 7th amino acids in this sequence were mutated from QN to ED as indicated with underline); **J**, 5'-CAG TGT GTC ATC CAC ATT TTC GAA AAT TTC CTT GGC-3 (SEQ ID NO:15) (corresponding to amino acid residues 10 38-27 in human protein C, the 7th amino acids in this sequence were mutated from Q to E as indicated with underline); **K**, 5'-GCC AAG GAA ATT TTC GAA AAT GTG GAT GAC ACA CTG-3' (SEQ ID NO:16) (corresponding to amino acid residues 27-38 in human protein C, the 6th amino acid in this sequence was mutated from Q to E as indicated with underline);

15 Both bovine and human protein C full length cDNAs were cloned into the Hind III and Xba I site of the vector pRc/CMV. To obtain bovine protein C mutant E33D34, PCR amplification of the target DNA was performed as follows. Bovine protein C cDNA containing the 5' terminus to the amino acid at position 40, was amplified with intact bovine protein C cDNA and primers A and C. The PCR reaction conditions were as described in  
20 Example 4. The sample was subjected to 30 cycles of PCR consisting of a 2 min denaturation period at 94°C, a 2 min annealing period at 55°C and a 2 min elongation period at 72°C. After amplification, the DNA was electrophoresed through an 0.8% agarose gel in 40 mM Tris-acetate buffer containing 1 mM EDTA. The PCR products were purified with The Geneclean III kit (BIO 101, Inc. USA), and the PCR fragment of bovine protein C  
25 cDNA containing the respective mutations was cleaved by Hind III and Bbs I. The Hind III/Bbs I fragment and the human protein C fragment (Bbs I - 3' terminus) were cloned into the Hind II and Xba I sites of pRc/CMV vector to produce a full-length bovine protein C cDNA with the mutations. Bovine protein C mutant H11 E33 D34 was created in the same way, but used bovine protein C mutant H11 as a template in the PCR reaction.

Human protein C cDNA containing the 5' terminus to amino acid-38 was PCR amplified with intact human protein C cDNA and primers A and D. Human protein C cDNA from amino acid 27 to the 3' terminus was amplified with intact human protein C cDNA and primers B and E. These two cDNA fragments were used as templates to amplify full length 5 bovine protein C cDNA containing mutated amino acids (E33 D34) with primers A and B. Human protein C mutant E33 was obtained by the following steps: human protein C cDNA containing the 5' terminus to amino acid 38 was amplified with intact human protein C cDNA and primers A and F. Human protein C cDNA from amino acid 27 to the 3' terminus was amplified with intact human protein C cDNA and primers B and G. These two cDNA 10 fragments were used as templates to amplify full length bovine protein C cDNA containing mutated amino acids (E33) with primers A and B. The PCR mixture and program were described above. The human protein C PCR products containing respective mutations were cleaved by Hind III and Sal I, and then the fragment (Hind III - Sal I) together with intact human protein C fragment (Sal I - 3' terminus) were cloned into the Hind III and Xba I sites 15 of pRc/CMV vector to produce the full length human protein C cDNA with the respective mutations. All mutations were confirmed by DNA sequencing prior to transfection.

The adenovirus-transfected human kidney cell line 293 was cultured and transfected as described in Example 4. Bovine and human recombinant protein C and mutants were purified as described in Example 4.

20 The vitamin K-dependent proteins were classified into four groups on the basis of their affinities for a standard membrane (Table 4). Sequences of the amino terminal residues of some relevant proteins including human protein C (hC), bovine protein C (bC), bovine prothrombin (bPT), bovine factor X (bX), and human factor VII (hVII) are given for reference, where X is Gla ( $\gamma$ -carboxyglutamic acid) or Glu.

25

**bPT:** ANKGFLXXVRK<sub>11</sub>GNLXRXCLXX<sub>21</sub>PCSRXXAFXA<sub>31</sub>LXSLSATDAF<sub>41</sub>WAKY (SEQ ID NO:17)  
**bX:** ANS-FLXXVKQ<sub>11</sub>GNLXRXCLXX<sub>21</sub>ACSLXXARXV<sub>31</sub>FXDAXQTDXF<sub>41</sub>WSKY (SEQ ID NO:18)  
**hC:** ANS-FLXXLRH<sub>11</sub>SSLXRXCIXX<sub>21</sub>ICDFXXAKXI<sub>31</sub>FQNVDDTLAF<sub>41</sub>WSKH (SEQ ID NO:1)  
**bC:** ANS-FLXXLRP<sub>11</sub>GNVXRXCSXX<sub>21</sub>VCXFXXARXI<sub>31</sub>FQNTXDTMAF<sub>41</sub>WSFY (SEQ ID NO:2)

**hVII: ANA-FLXXLRP11GSLRXCKXX21QCSFXXARXI31FKDAXRTKLF41WISY (SEQ ID NO:3)**

He had a very good time, and when he got home he told his mother all about it.

TABLE 4  
Charges and Affinity

	Residue						Total	$K_D$ (nM)
	11 +	29 +	33 +	34 = Sum				
<b>Class I</b>								
bZ	-2	+	-2	-1	-4	-6	0.2 <sup>a</sup> -32	
hZ	-2	+	-2		-3	-5	2.0 <sup>a</sup> -170	
<b>Class II</b>								
bPT-TNBS			-2		-2	-1	<10	
hVII-Q11E33		+	-2	-1	-2	-2	10	
hS		+	-2	-1	-2	-2	40	
bX		+	-2	-1	-2	-3	40	
bC-E33D34	P	+	-2	-1	-2	-4	125	
hX	+	+	-2	-1	-1	-2	160	
bPT	+		-2		-1	0	100	
hPT	+		-2		-1	-1	-	
bS	+	+	-2		0	0	120	
<b>Class III</b>								
bIX		+	-2		-1	-1	1000	
hIX		+	-2		-1	-1	1000	
hC		+			+1	-2	660	

<b>bC-H11</b>		+			<b>+1</b>	<b>-1</b>	930
<b>Class IV</b>							
<b>hC</b>	<b>P</b>	<b>+</b>			<b>+1</b>	<b>-2</b>	<b>3300</b>
hVII	P	+	+	-1	+1	+1	4000
bC	P	+			+1	-1	9200
bVII	P	+	+		+1	0	15000

<sup>a</sup> Higher affinity value equals  $k_{\text{dissociation}}/1*10^7 M^{-1} s^{-1}$ ; the denominator is a typical  $k_{\text{association}}$  for other proteins.

5 In Table 4, vitamin k-dependent polypeptide mutants are in bold. The total charge (residues 1-34) includes 7 calcium ions (+14) and the amino terminus (+1).

Protein Z was assigned to class I on the basis of its dissociation rate constant, which was 100 to 1000 times slower than that of other proteins. If protein Z displayed a normal association rate constant (about  $10^7 M^{-1} s^{-1}$ ) the  $K_D$  would be about  $10^{-10} M$ . Wei, G.J.

10 et al., 1982, Biochemistry, 21:1949-1959. The latter affinity may be the maximum possible for the vitamin K proteins. Class IV proteins differed from class III in the presence of proline-11, which may alter affinity by non-electrostatic means.

While a relatively weak correlation existed between membrane affinity and net negative charge on residues 1-34, an excellent correlation was found when only residues 5, 15 11, 29, 33 and 34 were considered (Table 4). The latter amino acids are located on the surface of the protein. A number of proteins were modeled by amino acid substitution into the prothrombin structure and their electrostatic potentials were estimated by the program DelPhi. A sketch patterned after the electrostatic potential of bovine protein Z is shown in Figure 11. Electronegative sites at 7, 8, 26, 30, 33, 34 and 11 produce a halo of 20 electronegative charge surrounding a cationic core produced by the calcium-lined pore (Figure 11). The closer a protein structure approaches this structure, the higher its affinity for

the membrane. This correlation is apparent from the wild-type proteins, the mutants and chemically modified proteins.

The pattern for other structures can be extrapolated from examination of the charge groups that are absent in other proteins. For example, Lys-11 and Arg-10 of bovine 5 prothrombin generate high electropositive regions in their vicinity; the lack of Gla-33 in protein C and Factor VII create less electronegativity in those protein regions. In all cases, highest affinity corresponded to a structure with an electropositive core that was completely surrounded by electronegative protein surface, as shown for protein Z. The exceptions to this pattern are the proteins with Pro-11, which may lower affinity by a structural impact and ser-10 12 (human protein C), which is a unique uncharged residue.

To further test the hypothesis of an archetype for electrostatic distribution, site-directed mutagenesis was used to replace Gln33Asn34 of bovine and human protein C with Glu33Asp34. Glu33 should be further modified to Gla during protein processing. These changes altered the electrostatic potential of bovine protein C to that of bovine factor X. The 15 membrane affinity of the mutant protein was expected to be lower than that of factor X due to the presence of proline-11. Indeed, the bovine protein C mutant gave a membrane affinity similar to that of bovine prothrombin (Figure 12A), and slightly less than that of bovine factor X (Table 4).

More interesting was that clot inhibition by APC was greater for the mutant than 20 for the wild type enzyme (Figure 12B, C). Inclusion of results for the P11H mutant of bovine protein C from Example 3 showed that a family of proteins could be produced, each with different membrane affinity and activity, by varying the amino acid substitutions at positions 11, 33 and 34.

Human protein C mutants containing E33 and E33D34 resulted in a small 25 increase in membrane binding affinity (Figure 13a). Activity of these mutants was slightly less than the wild-type enzyme (Figure 13b). Results with mutants of bovine protein C suggest that failure of the E33D34 mutation in the human protein may arise from H11 and/or other unique amino acids in the protein. Figure 14A shows that the H11 mutant of bovine protein C bound to the membrane with about 10-fold higher affinity than wild type protein,

the E33D34 mutant bound with about 70-times the affinity, but that the triple mutant, H11E33D34, was only slightly better than the H11 mutant. This relationship was mirrored in the activity of APC formed from these mutants (Figure 14B). This result suggested that the presence of H11 reduced the impact of E33D34 on membrane binding affinity.

5 These results indicated that introduction of E33D34 may not be optimal for all proteins. Consequently, other mutations may be desirable to create human protein C that will use E33D34 and have maximum increased membrane affinity. The result with the bovine protein suggested that histidine 11 may be the primary cause of this phenomenon. Consequently, H11 may be altered to glutamine or to another amino acid in human protein C, 10 along with the E33D34 mutation. Another amino acid that may impact the affinity is the serine at position 12, an amino acid that is entirely unique to human protein C. These additional changes should produce proteins with enhanced membrane affinity.

15 The electrostatic archetype was also tested by comparison of human and bovine factor X. The presence of lysine-11 in human factor X suggests that it should have lower affinity than bovine factor X. This prediction was borne out, by the result shown in Figure 15.

20 Earlier studies had shown that trinitrobenzenesulfonic acid (TNBS) modification of bovine and human prothrombin fragment 1 had relatively little impact (0 to 5-fold) on membrane affinity. Weber, D.J. et al., 1992, *J. Biol. Chem.*, 267:4564-4569; Welsch, D.J. et al., 1988, *Biochemistry*, 27:4933-4938. Conditions used for the reaction resulted in derivatization of the amino terminus, a change that is linked to lowered membrane affinity. Welsch, D.J. and Nelsestuen, G.L., 1988, *Biochemistry*, 27:4939-4945. Protein modification in the presence of calcium, which protects the amino terminus, resulted in TNBS-modified protein with much higher affinity for the membrane than native fragment 1.

25 The suggestion that protein Z constitutes the archetype was based on its dissociation rate constant and that a normal association rate would generate a  $K_D=10^{-10}$  M. Whether this value can be reached is uncertain. It is possible that the slow association rate of protein Z is caused by improper protein folding, resulting in a low concentration of the membrane-binding conformation. If conditions can be altered to improve protein folding,

association rates of protein Z should improve. Indeed, the association rate constant for protein Z was improved by alteration of pH. The basis for this observation may be related to an unusual feature of the prothrombin structure which is the close placement of the amino terminus (+1 at pH 7.5) to calcium ions 2 and 3. The +1 charge on the amino terminus is 5 responsible for the slight electropositive region just above Ca-1 in Figure 11. Charge repulsion between Ca and the amino terminal may destabilize protein folding and could be a serious problem for a protein that had low folding stability.

Table 5 provides additional support for the archetype model. It shows the relationship between distance of ionic groups from strontium ions 1 and 8 (corresponding to 10 calcium 1 and an extra divalent metal ion found in the Sr x-ray crystal structure of prothrombin). The pattern suggests that the closer an ionic group is to these metal ions, the higher its impact on membrane affinity. The exception is Arg-16, which contributes to the charge of the electropositive core. Higher affinity is correlated with electronegative charge at all other sites. This correlation also applies to the GLA residues.

15

**TABLE 5**  
**Distance to Sr-1, 8 and Ion Importance.**

		Distance (Å) to:		Impact/ion
Position	Atom <sup>a</sup>	Sr-1	Sr-8	on K <sub>D</sub> (K <sub>M</sub> )
(A.A-Protein)				
3(K-PT)	$\varepsilon$ -N	22.1	21.7	Low or Unknown
5(K-IX)	para-C(F)	20.1	20.8	"
19(K/R-VII)	C5(L)	20.2	17.8	"
22(K-IX)	C4(P)	17.0	18.5	"
10(R)	C6(R)	16.8	12.9	"
25(R-PT)	C6(R)	11.2	13.8	"
24(X/D-PC)	O(S)	8.1	12.0	"
11(K-PT,hX,bS; Gla-PZ)				
	$\varepsilon$ -C(K)	14.7	7.4	3-10-fold <sup>b</sup>
33(Gla)	$\gamma$ -C(E)	11.6	7.5	"
34(D)	O(S)	15.3	12.1	"
29(R)	para-C(F)	7.5	8.4	"
16(R)	C6(R)	14.2	10.6	" <sup>c</sup>
Gla residue <sup>d</sup>				
Low importance:				
7		12.8	13.3	+2 (<2)

15		20	16	<2 (<2)
20		19.4	17.8	<2 (<2)
21		17.2	15	4(3)
33		11.6	7.5	? <sup>e</sup> (<2)
High importance:				
8		8.7	10.9	? <sup>e</sup> (20)
26		3.6	9.5	? <sup>e</sup> (50)
17		11.1	9.1	>200(100)
27		8.4	10.6	>200(85)
30		3.4	4.2	>200(25)

<sup>a</sup> Distances are from this atom of bovine prothrombin (residue of prothrombin used in measurement is given in parentheses) to strontium 1 and 8 of the Sr-Prothrombin fragment 1 structure. Seshadri et al. 1994, Biochemistry

5 33:1087-1092. <sup>b</sup> For all but 16-R, cations lower affinity and anions increase affinity.

<sup>c</sup> Thariath et al. 1997 Biochem. J. 322:309-315. <sup>d</sup> Impact of Glu to Asp mutations, distances are averages for the gamma-carboxyl-carbons.  $K_D(K_M)$  data are from Ratcliffe et al. 1993 J. Biol. Chem. 268:24339-45. <sup>e</sup> Binding was of lower capacity or caused aggregation, making comparisons less certain.

The results in Figure 15C show that the association rate for protein Z was 10 substantially improved at pH 9, where an amino terminal should be uncharged. The rate constant obtained from these data was about 12-fold higher at pH 9 than at pH 7.5 (Figure 15C).

#### Other Embodiments

15 It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.